Rat colitis induced by intrathecal injection of substance P

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Abstract: The aim of this study was to, from the point of neurogenic inflammation, explore the pathogenesis of colitis and to provide direct evidence for the neurogenic colitis hypothesis. Male Sprague-Dawley rats (180-220 g) anesthetized with chloral hydrate were intrathecally (ith) implanted with polyethylene-10 (PE-10) catheter to reach the spinal cord T12-L5 level. Substance P (SP) was ith injected once a day for 14 d. The disease active index (DAI) score was calculated by rat body weight and stool. The macroscopic and HE staining-microscopic pathologies of colon/spinal tissue were evaluated. By immunofluorescence staining, the protein expression of a pro-inflammatory cytokine, migration inhibitory factor (MIF), in colon tissue was detected and was semi-quantitatively analyzed.

The results showed that in the colon tissue, inflammation was dose-dependently aggravated by ith SP 10 μg and 20 μg, whereas in the spinal tissue, only slight edema and congestion were seen in SP 20 μg group. The MIF protein of colon tissue was increased in ith SP 10 μg and 20 μg groups (P<0.05, P<0.01 as compared to normal saline group respectively), but in the spinal tissue, there was no obvious MIF protein expression either in SP groups or in normal saline group. Pretreatment with neurokinin-1 (NK1) receptor antagonist ([D-Pro2, D-Trp7, 9]–SP, 22.4 μg, ith, 10 min before ith SP) prolonged the latency of DAI rising and reduced the DAI amplitude, as well as prevented the high MIF expression induced by ith SP. These results suggest that rat colitis can be induced by direct SP stimulation in lumbar spine via activating central NK1 receptor; and that colonic MIF is possibly one of the inflammatory factors involved in this pathogenesis. These data provide a reasonable support to the hypothesis of colitis being a neurogenic inflammation. In addition, a potential clinical significance for the finding that higher concentration of spinal SP can induce colitis via NK1 receptor is discussed.

Key words: colitis; neurogenic inflammation; spinal cord; substance P; NK1 receptor; rats

脊髓蛛网膜下腔注射 P 物质引起大鼠结肠炎

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摘 要: 本实验从神经源性炎症的角度探讨结肠炎(colitis)发病的机理，并为神经源性结肠炎的假说提供直接的证据。健康雄性 Sprague-Dawley 大鼠(180-220 g)经水合氯醛腹腔麻醉后,将 PE-10 管插入脊髓蛛网膜下腔达到 T12-L5 水平。鞘内(intrathecal, ith)注射 P 物质(substance P, SP), 每天一次, 共 14 天。神经激肽-1 (neurokinin-1, NK1) 受体拮抗剂([D-Pro2, D-Trp7, 9]–SP, 22.4 μg) 于每次 ith 注射 SP 前 10 min ith 预处理, 观察疾病活动指数(disease active index, DAI)、结肠和脊髓组织的大体 / 镜下病理, 以及移动抑制因子(migration inhibitory factor, MIF)蛋白的表达。结果显示: ith 注射 SP 10 μg 和 20 μg 能够引起大鼠 DAI 明显升高, 结肠炎症浸润、腺体萎缩和 MIF 高表达(与生理盐水组相比, P<0.05, P<0.01); 但在脊髓, 仅在 ith 注射 SP 20 μg 组见到轻度充血水肿, 并无明显神经元坏死。NK1 受体拮抗剂预处理, 能够延长 ith 注射 SP 20 μg 后 DAI 开始升高的潜伏期, 减小 DAI 升高的幅度, 抑制 MIF 在结肠组织的高表达。上述实验结果表明, ith 注射 SP 能够引起大鼠结肠炎, 该效应是通过激活背侧 NK1 受体实现的, 肠组织的 MIF 参与了此炎症过程。脊髓高浓度 SP 能通过 NK1 受体介导结肠炎这一发现可能具有潜在的临床意义。

关键词: 结肠炎; 神经源性炎症; 脊髓; P 物质; 神经激肽-1 受体; 大鼠

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Ten years ago, Barbara et al. advanced a challenging hypothesis that human colitis may be one of the neurogenic inflammations in the gut\(^1\). This hypothesis opened up a new way for exploring the pathogenesis of colitis. The original definition for neurogenic inflammation is that by stimulation of peripheral nerves, the release of pro-inflammatory neuropeptides promotes plasma leakage from the postcapillary venules. The local tissues display increased blood flow and neutrophil infiltration, an inflammatory process. This process usually occurs in skin, airways, and pelvic cavity\(^2,3\). Substance P (SP) and neurokinin-1 (NK\(_1\)) receptor were reported to be involved in this process\(^4\). Since Barbara put forward his suggestion on neurogenic colitis, several laboratories have, from different points of view, provided mechanism connections between colitis and neurogenic inflammation, one of which was the blockade of the NK\(_1\) receptor\(^5\). But so far, the cause-effect relationship between colitis and neurogenic inflammation is still short of direct evidence. Recently we reported that by intrathecal (ith) injection of a hapten to rat lumbar spinal cord, an inflammation in the colon was seen\(^6,7\), thus providing an operative animal model for exploring the possibility of spinal nervous system affecting the colon via stimulation of the spinal cord. To our knowledge, SP is an important pro-inflammatory peptide as well as a neurotransmitter in spinal and enteric nervous system. In addition, it is reported that NK\(_1\) receptor was up-regulated in patients with colitis\(^8\) and that colitis could be protected by NK\(_1\) receptor antagonist in human and animal, suggesting a role for SP in the development of intestinal inflammation\(^5,9\). In Barbara’s hypothesis, SP was particularly emphasized to involve in the root ganglia antidromic stimulation-induced colitis relapse\(^10\). However his hypothesis has not been testified. In the present study, we would like to, with the animal model mentioned above\(^6,7\), explore whether rat colitis would happen when enteric transmission was activated by stimulating its spinal center with SP, and what the initial mechanisms for the ith SP-induced colitis would be.

1 MATERIALS AND METHODS

1.1 Animal and drugs

Male Sprague-Dawley rats (180-220 g, Grade II) were obtained from the Department of Experimental Animals, Peking University Health Science Center. Rats were fed with a standard diet and allowed free access to water. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council, National Academy Press, Washington, D.C. 1996). SP and NK\(_1\) receptor antagonist ([D-Pro2, D-Trp7, 9] -SP) were bought from Sigma. Rabbit anti-rat migration inhibitory factor (MIF) multicleon antibody was bought from Santa Cruz. Mouse anti-human (and rat) microtubule associated protein 2a, b, c (MAP2a, b, c) monoclonal antibody was bought from ZYMED. TRITC (tetraethyl rhodamine isothiocyanate)-labeled goat anti-rabbit IgG and FITC (fluorescein isothiocyanate) labeled goat anti-mouse IgG were bought from ZACKSON (USA). Polyethylene-10 (PE-10) catheter was bought from PORTEX Limited.

1.2 Drug administration and ith instillation

As described by original literature\(^10\) with our modifications\(^9\), a PE-10 catheter was inserted into the spinal subarachnoid space to reach T\(_{12}\)-L\(_{5}\) level after the rats were anesthetized with intraperitoneal (i.p.) injection of chloral hydrate (0.3 g/ kg). Ten thousand unit penicillin was i.p. injected to prevent infection. All the drug administration began three days later. SP was dissolved with normal saline (NS) to the concentration of 1 mg/mL. In ith SP experiment, 32 rats were randomly divided into 4 groups. From the first day of experiment SP was respectively ith injected at 5 \(\mu\)g, 10 \(\mu\)g and 20 \(\mu\)g once a day for 14 days. The control group was ith injected with NS 10 \(\mu\)L. NK\(_1\) receptor antagonist was dissolved with minimum volume NS and then filled up with PBS to final concentration of 15.0 mmol/L. In NK\(_1\) receptor antagonist pretreatment experiment, 24 rats were randomly divided into 3 groups. NK\(_1\) receptor antagonist 11.2 \(\mu\)g or 22.4 \(\mu\)g was ith injected respectively once a day 10 min prior to each SP 20 \(\mu\)g ith injection. The control group was ith injected with PBS 10 \(\mu\)L. On the 15th day of experiment, all the rats were killed by decapitation.

1.3 Disease activity index (DAI) score and pathological evaluation

After ith injection of SP the rat body weight and stool were daily observed by which the DAI was scored as described elsewhere in detail\(^11\). Briefly, the body mass and stool were scored as follows: Body mass: score 0 — normal; score 1 —1%-5% lower than normal; score 2 — 6%-10% lower than normal; score 3 — 11%-15% lower than normal; and score 4 — above 15% lower than normal. Stool viscosity: score 0 — normal; score 2 — fluffy; and score 4 — diarrhea. Stool hemorrhage: score 0 — normal; and score 2 — apparent hemorrhage. Soon after the rat was killed the tissues
(dissected out from the inflammatory colon and around the tip of PE-10 catheter) were first macro-pathologically observed and then placed in 10% formaldehyde overnight. Paraffin-embedded section (5-µm thick) for haematoxylin and eosin (HE) staining were followed by microscopic observation and evaluation.

1.4 Immunohistochemistry of MIF
By using a modification of the method originally described in rat[12], the paraffin-embedded colon and spinal samples were treated with deparaffinage, hydration, antigen reparation and blocking the non-specific binding. For double immunofluorescence staining, anti-MIF multiclone antibody and anti-MAP2 a,b,c monoclon antibody (1:300) were added simultaneously to the sample. Twelve hours after incubation at 4 ºC, TRITC-labeled and FITC-labeled secondary antibody (1:300) were added simultaneously to the sample. For single immunofluorescence staining, only anti-MIF multiclone antibody (1:300) was added to the sample followed by 12 h incubation at 4 ºC, and then TRITC-labeled secondary antibody (1:300) was added to the sample. All the samples were incubated at room temperature for 60 min (away from light). Photographs were obtained using a fluorescence microscope (Nikon-Eclipse TE2000-µ, Japan). MIF protein fluorescence intensity was observed and was semi-quantitively analyzed by LEICA Qwin software.

1.5 Statistical methods
For all statistical procedures, PRISM 4.0 software was used. Quantity data were expressed as mean±SD. One-way ANOVA and t-test were used to evaluate significant differences between the groups. Generally, P<0.05 was considered statistically significant.

2 RESULTS

2.1 Pathological changes in rat colon and spinal cord by ith SP
Macropathologically, in the ith SP 10 µg and 20 µg groups, the distension of enteric cavity and the adhesions of mucous membrane were seen. In the spinal cord no macro-pathological change was seen.

Under microscope, in the SP 10 µg and 20 µg groups, significant lymphocyte infiltrations in colonic mucosa and submucosa were observed. Atrophies in both mucosa and gland were also noted. The ith SP 5 µg group had normal histological structures as in the control group. Pretreatment of NK1 receptor antagonist (11.2 µg) partly inhibited the evident atrophy of glands. The inflammation of colonic tissue was completely inhibited by 22.4 µg of NK1 receptor antagonist (Fig. 1). In the spinal tissue, a slight basophilic change of neurons was seen in ith SP 20 µg group.

Fig. 1. The histological changes of rat colon by ith injection of SP (HE staining). A: Control (NS 10 µL) group, normal histological structure. B: SP 5 µg group, basically normal histological structure. C: SP 10 µg group, mucosa atrophy and lymphocyte infiltration. D: SP 20 µg group, lymphocyte infiltration and evident atrophy of glands. E: NK1 receptor antagonist (11.2 µg) + ith SP 20 µg group, mucosa edema and much lymphocyte infiltration. F: NK1 receptor antagonist (22.4 µg) + ith SP 20 µg group, normal in histology. Scale bar, 100 µm.
Fig. 2. The histological changes of rat spinal cord by i.th injection of SP (HE staining). A: Control (NS 10 µL) group, normal histological structure. B: SP 20 µg group, tissue congestion. C: Control (NS 10 µL) group, a distinct constitution and regular arrangement in neuron. The substantia alba had a uniform staining. D: SP 20 µg group, a slight basophilic change was seen in some neurons. Scale bar, 100 µm in A and B, 200 µm in C and D.

Fig. 3. Expression of MIF protein in rat colon tissue by i.th SP. A: Control (i.th PBS + i.th NS 10 µL), little red fluorescence spot was seen. B: i.th PBS + i.th SP 5 µg, little red spot was seen. C: i.th PBS + i.th SP 10 µg, an increased red spot was seen. D: i.th PBS + i.th SP 20 µg, there was stronger red fluorescence than that in A, especially around the glands. E: i.th NK1 receptor antagonist + i.th SP 20 µg, the red spots were less than those in D. NK1 receptor antagonist (22.4 µg) or PBS (10 µL) was injected 10 min prior to i.th SP. Single immunofluorescence staining. Scale bar, 100 µm.

Fig. 4. Expression of MIF protein in rat spinal cord by i.th SP. A: i.th NS 10 µL. B: i.th SP 5 µg. C: i.th SP 10 µg. D: i.th SP 20 µg. There were no obvious differences in MIF expression among the four groups. Single immunofluorescence staining. Scale bar, 100 µm.
In addition, the axon root was thickening and the substan-
tia alba was puff and edema in ith SP 20 µg group. There
was no evident neuron necrosis (Fig. 2).

2.2 MIF protein expression in colon and spinal cord
by ith SP
Colonic MIF protein expression (red fluorescence spot)
was not increased by ith SP 5 µg as compared to the con-
trol group (Fig. 3, B vs A). In ith SP 10 µg and 20 µg
groups, the MIF expression was much higher than that in
the control group (Fig. 3, C vs A, D vs A). NK₁ receptor
antagonist prevented the SP-induced higher MIF expres-
sion (Fig. 3, E vs D). In spinal cord, SP (5 µg, 10 µg and
20 µg) did not induce an obvious expression of MIF pro-
tein (Fig. 4A-D).

Semi-quantitative analysis showed that there were sig-
nificant differences in MIF expression between ith SP (10,
20 µg) groups and control group in colon tissue (Table 1),
but there were no significant differences in MIF expres-
sion between ith SP groups and control group in spinal
cord (Table 2).

<table>
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<th>Group</th>
<th>MIF fluorescence intensity</th>
<th>Mean±SD</th>
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<tr>
<td>NS 10 µL</td>
<td>70.17</td>
<td>72.64</td>
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<td>SP 5 µg</td>
<td>79.11</td>
<td>72.41</td>
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<tr>
<td>SP 10 µg</td>
<td>90.16</td>
<td>95.44</td>
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<tr>
<td>SP 20 µg</td>
<td>119.14</td>
<td>96.53</td>
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MIF: migration inhibitory factor; ith: intrathecal; SP: substance P. MIF protein fluorescence intensity was semi-quantitively analyzed by LEICA Qwin software. *P<0.05, **P<0.01 vs NS group.

<table>
<thead>
<tr>
<th>Group</th>
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<th>Mean±SD</th>
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<tr>
<td>NS 10 µL</td>
<td>87.06</td>
<td>168.71</td>
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<td>SP 5 µg</td>
<td>102.18</td>
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<td>SP 10 µg</td>
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<td>88.15</td>
</tr>
<tr>
<td>SP 20 µg</td>
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MIF: migration inhibitory factor; ith: intrathecal; SP: substance P. MIF protein fluorescence intensity was semi-quantitively analyzed by LEICA Qwin software. There were no statistical differences among the 4 groups.

2.3 NK₁ receptor antagonist delayed the DAI eleva-
tion and prevented the MIF expression induced by
ith SP
Pretreatment of NK₁ receptor antagonist (ith) could pro-
long the latency of DAI rising and reduce the DAI amplti-
dude induced by ith SP 20 µg. On day 15, the difference
of DAI score between NK₁ receptor antagonist pretreatment
group and PBS pretreatment group was statistically sig-
nificant with the P value less than 0.01 (Fig. 5). In addition,
the MIF expression in NK₁ receptor antagonist pretreat-
ment group was lower than that in PBS pretreatment group
(Fig. 3, E vs D).

3 DISCUSSION
For the first time we have given the verification in this paper that if rat spinal segment innervating the gut was
stimulated by exogenous SP in pharmacological doses, colitis may be induced. From the experimental procedure, it would be that the injected SP first activated the spinal neurons and then, via spinal efferent pathway, resulted in the colitis. This reaction pattern provided a more persuasive model for studying the neurogenic mechanism of colitis.

Concerning the interrelations of neuritis and colitis, there have been published data from different points of view to support Barbara’s hypothesis, such as blocking the nerve impulse[13], exsecting the innervations[14], depleting the neurotransmitters[15] and competitively binding the receptors[13, 16, 17], however due to these data either came from the patients who had been diagnosed as colitis before they were enrolled in the study[13, 16] or came from animal models in which the colons were stimulated beforehand and the nerves were treated/observed afterward[14, 15, 17, 18], the cause-effect relation of colitis-neuritis is still ambiguous. SP was implicated in the neurogenic colitis by Barbara[11], which mainly came from a clinical observation that spinal cord stimulation by an electrode positioned on patient skin at the level of the dorsal aspect of the lower spinal cord produced successive relapses of colitis[19]. Barbara supposed that SP released by the spinal cord stimulation led to the colitis relapses[11]. However, there is no concrete experiment to demonstrate his assumption. In the other papers (including the finding that colonic SP nerve density/morphology were changed in colitis patients[20], a shift from mainly cholinergic to more SP positive innervations was seen in the myenteric neurons of colitis rats[21]; and SP precursor β-preprotachykinins in rat neurons of myenteric plexus was transcribed faster in an acute phases of colitis[22], etc), SP was at most suggested to involve in colitis, but could not be certificated to act directly as a neurogenic stimulator for colitis occurring.

So in our present study, we focused on the efferent action of enteric nerves by central stimulation with SP. It would be more directly and concisely to expound Barbara’s conjecture for SP inducing colitis relapse by the root ganglia antidromic stimulation[13]. We found that after ith administration of SP, the spinal tissue displayed neuritis (tissue congestion, neurons basophilic change), but no visible necrosis was found because the Nissl body was still able to be identified and the axon still existed (Fig. 2). At the same time the colon pathological change was much obvious (Fig. 1) and the colonic MIF expression was significantly increased by ith SP (Fig. 3, Table 1), which points out that a colitis was induced by spinal neuritis. After pretreatment with NK1 receptor antagonist, the DAI score significantly decreased (Fig. 5) and the colonic MIF expression was much less than PBS pretreatment group (Fig. 3), which points out that the ith SP-induced colitis is mediated by spinal NK1 receptor. It is well established that NK1, NK2 and NK3 receptors are presented in the spinal cord of rat[23]. So it will be interesting to further study and compare the functions of the three kinds of NK receptors in the ith SP-induced colitis.

Contrasting to our recent report that DNCB (a hapten) could induce rat inflammation in spinal/colonic tissues and higher MIF expression in spinal/enteric neurons[24, 25], ith SP neither induced obvious MIF expression in enteric/spinal neurons (immunofluorescence double staining, photographs were not shown here) nor induced the mucosal erosion, necrosis and ulcer of colon. SP induced the mucosal atrophy and lymphocyte hyperplasia in colon which represented a chronic colitis. These disparities suggested that different mechanisms for different stimulators induced spinal activation and colitis. Moreover, it would be worth mentioning that ith SP-induced colitis model in normal rats (here) may have more potential clinical significance than ith DNCB-induced colitis model in pre-sensitized rats[26, 27]. Based on recent finding that psychological stress which had been mentioned to induce colitis[24] may activate brain SP transmission because ith or icv NK1 receptor antagonist could attenuate the anxiety[25, 26], we hypothesized that ith SP (here) may, to some extent, mimic a situation of anxiety activated by NK1 receptor in spinal tissue. So we expect that oral administration of NK1 receptor antagonist that was centrally active[27] will be convenient and reasonable for not only treating anxiety but also preventing colitis.

There have been reports that the spinal NK1 receptors were abundant in the dorsal horn and appeared mainly of dendrite nature[28], SP immunoreactive axonal buttons are preferentially presynaptic to neurons expressing the SP receptor[29], suggesting a short-distance action of SP in the spinal cord. Undoubtedly, ith SP first activated the spinal NK1 receptor and then induced the colonic inflammation, but a long-distance transmission to its target (colon) would need other transmitters and intermedial pathway.

Results from the present study establish that rat colitis can be induced by direct lumbar stimulation with SP which could only induce slight spinal inflammation but did not destroy the neurons. Central NK1 receptor mediated the SP-induced colitis. Colonic MIF was also involved in the neuritis-induced colitis process. It would deserve to explore other central mediators in this chronic ith SP model. Sun et al.[30] reported that by acute ith NK1 receptor agonist to the rat, an increased expression of nitric oxide synthases (NOS) and increased production of nitric oxide in
the lumbar spine were found, from which we suppose that maybe in the earlier days of ith SP, the NOS, even the MIF, in spinal cord was activated, while in the later days of ith SP, MIF showed obsolete activity. It would also be deserved to further explore other peripheral proinflammatory factors involved in this process except MIF.

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REFERENCES


